PLATELET CYTOCHROME P-450: A POSSIBLE ROLE IN ARACHIDONATE-INDUCED AGGREGATION

D. L. Cinti and M. B. Feinstein

Department of Pharmacology
University of Connecticut Health Center
Farmington, Connecticut 06032

Received September 3,1976

SUMMARY: Platelet microsomes were shown to contain cytochromes P-450 and b_5 and their respective reductases, NADPH-cytochrome c reductase and NADH-cytochrome b_5 reductase. Metyrapone and carbon monoxide (CO), two inhibitors of cytochrome P-450, inhibited both the arachidonic acid-induced platelet aggregation and the formation of aggregating factors from arachidonic acid by isolated microsomes. In addition metyrapone produced a type II spectral change with platelet microsomal cytochrome P-450. The data suggest that cytochrome P-450 may play a role in the complex enzyme systems which convert arachidonic acid to the platelet aggregating factors, cyclic endoperoxides and thromboxane A_2.

INTRODUCTION

A detailed understanding of the enzymes and cofactors necessary for catalyzing the transformations of arachidonic acid to platelet aggregating factors is lacking. A cyclooxygenase (1), present in microsomes (2,3), is involved in the initial metabolic steps in the conversion of arachidonic acid to PGG_2 and PGH_2. The prostaglandin endoperoxide synthetase of bovine vesicular gland microsomes which converts 8,11,14-eicosatrienoic acid to PGG_1 and PGH_1 probably consists of two enzyme activities (4), one of which is the cyclooxygenase. Both enzymes require the addition of free or protein-bound heme for their activity (4). A heme requirement for the platelet microsomal system has also been reported (2). Another platelet

---

Abbreviations

PGG_1, PGH_1, PGG_2, PGH_2 - prostaglandins G_1, H_1, G_2, H_2 respectively.

ADP - adenosine diphosphate.

microsomal enzyme, designated thromboxane synthetase (5,6), catalyzes the formation of highly labile thromboxane A_2 from PGG_2 or PGH_2; however little is known about the nature of this enzyme. The involvement of a peroxidase activity (3,7) in cyclic endoperoxide synthesis has also been suggested. Based on these observations we have investigated the platelet microsomal composition of heme proteins, especially cytochrome P-450, since this enzyme is normally localized to the endoplasmic reticulum of cells in which it occurs, can act as a peroxidase (8,9), catalyzes the insertion of oxygen into its substrates, and its reduced form may reduce aromatic hydrocarbon epoxides (10).

Our results show the presence in the platelet microsomal fraction of cytochrome P-450, cytochrome b_5 and their respective flavoprotein reductases. Metyrapone and CO were found to block both arachidonate-induced platelet aggregation and the generation by isolated platelet microsomes of platelet aggregating factors and rabbit aorta contracting substance (RCS) from arachidonate.

MATERIALS AND METHODS

Human platelet rich plasma, or platelet concentrates obtained from the Connecticut Red Cross Blood Center, Farmington, Conn. were prepared as previously described in detail (11). Platelet aggregation was monitored by recording light transmission in a modified Turner 350 spectrophotometer or a Chronolog aggregometer. The microsomal fraction, isolated after sonication (30 sec., 70 watts at 0°C; Branson Sonifier) of washed platelets, was obtained by centrifugation at 105,000 x g for 1 hour, after prior removal of the 10,000 x g (15 min.) pellet. The microsomes were resuspended in 0.1M Tris-HCl, pH 7.5 at a final protein concentration of approximately 15 mg/ml.

The generation of endoperoxides and thromboxanes from arachidonic acid in platelet microsomes was bioassayed on indomethacin (15μM) treated intact platelets and on indomethacin treated rabbit aorta strips (5,6).

Cytochromes P-450 and b_5 were measured with the Aminco DW-2 dual wavelength recording spectrophotometer as described by Omura and Sato (12). Spectral changes produced by the addition of metyrapone to a suspension of platelet microsomes were also recorded with the same spectrophotometer. NADPH-cytochrome c reductase activity was measured by the method of Phillips and Langdon (13). NADH-cytochrome b_5 reductase was determined by the method of Strittmatter (14). In some experiments both the 10,000 x g and 105,000 x g pellets were resuspended in 0.25M

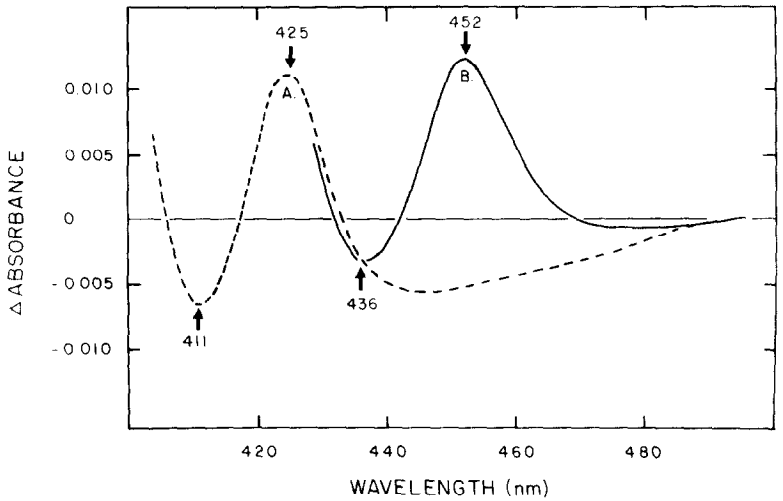

Figure 1. Difference spectra of NADH-reduced cytochrome b_5 and CO + dithionite-reduced cytochrome P-450 from human platelet 105,000 x g fraction ("microsomal"). Platelet microsome suspension (5.8mg protein/ml) in 0.1M Tris HCl, pH 7.4 was divided equally into two cuvettes, a baseline recorded and cytochromes b_5 and P-450 determined by the procedure of Omura and Sato (12).

sucrose and analyzed spectrally for mitochondrial cytochrome by the addition of 10mM succinate. Protein concentrations were determined by the method of Lowry et al. (15).

Arachidonic acid (Grade I), NADPH, NADH, cytochrome c and indomethacin were obtained from Sigma Chemical Co., St. Louis, Mo. Metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone was a product of Aldrich Chemical Co., Inc., Milwaukee, Wisc. A23187 was a gift from Eli Lilly Co., Indianapolis, Ind. Collagen was prepared from bovine achilles tendon (Sigma) by acetic acid extraction (16).

RESULTS

In the presence of the chemical reductant dithionite and CO platelet microsomes exhibited a difference spectrum uniquely characteristic of cytochrome P-450 (fig. 1). The absorption maximum in the Soret region was at 452nm identical to the peak obtained with kidney cortex microsomal cytochrome P-450 ($P\text{-}450_K$) (17). Also seen in figure 1 is the NADH-reduced cytochrome b_5 difference spectrum in which the absorbance maximum and minimum are identical to that of cytochrome b_5 found in other cells.

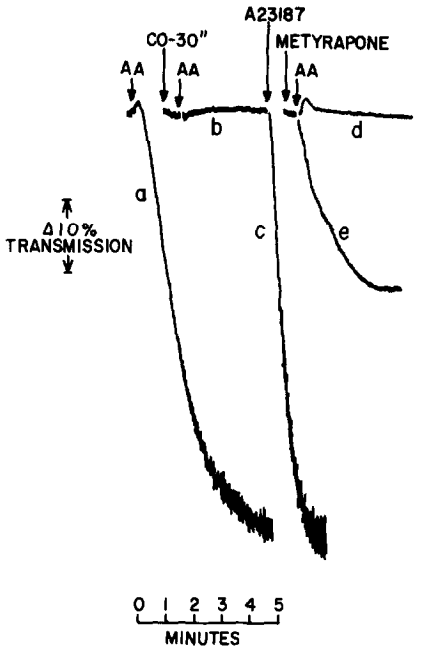

Figure 2. *Effects of CO and metyrapone on platelet aggregation induced by arachidonic acid (AA). (a) control, 35μM AA, (b) CO bubbled into platelet suspension for 30 sec., (c) A23187 (2μM) added to CO treated platelets, (d) platelet suspension + 1.2mM metyrapone, (e) + 0.6mM metyrapone.*

Platelet microsomes were also found to contain the flavoproteins, NADH-cytochrome c reductase which transfers reducing equivalents to cytochrome P-450, and NADH-cytochrome b_5 reductase. The specific content of platelet microsomal P-450 was 0.027nmols/mg protein and the specific activity of its flavoprotein was 3.3nmols/min/mg protein. The cytochrome b_5 content was 0.024 nmoles per mg protein and the NADH-cytochrome b_5 reductase activity was 2.6 μmoles/min/mg protein. The 10,000 x g particulate fraction contained only the mitochondrial cytochromes.

Since CO and metyrapone (18) are well known inhibitors of cytochrome P-450-dependent oxidative reactions, their effects on arachidonate-induced platelet aggregation were studied. Bubbling platelet suspensions with CO alone, or with CO plus oxygen at equal flow rates, for as little as 30 sec. completely abolished the arachidonate-induced aggregation (fig. 2).

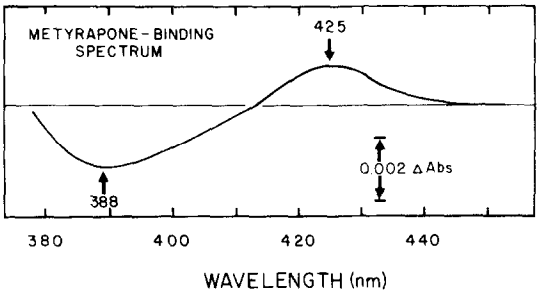

Figure 3. Type II spectral change produced by addition of metyrapone to platelet microsomes (2.4mg/ml) suspended in 50mM Tris buffer, pH 7.4. The suspension was divided equally into two cuvettes and a baseline of equal light absorbance was established. 20µl metyrapone (30mM in Tris HCl) was added to the sample cuvette to give a final concentration of 0.3mM, and the reference cuvette received 20µl of buffer.

However, normal aggregation was elicited under the same conditions by the Ca^{++} ionophore A23187, adenosine diphosphate (20µM), or high concentrations of collagen (20µg/ml). Treatment of the platelets with rotenone (5µM) or with nitrogen, rather than CO, for 5 minutes had negligible effects on the arachidonate-induced aggregation. The effect of CO was slowly reversed by replacement with air or O_2.

Metyrapone also rapidly inhibited arachidonate-induced aggregation (fig. 2), but not that due to A23187, ADP or a high concentration of collagen. Moreover, the addition of metyrapone to suspensions of platelet microsomes resulted in the appearance of a typical P-450 type II spectral change (fig. 3) characteristically observed with liver microsomes (19).

Our platelet microsomal preparations, when incubated with arachidonic acid, generated endoperoxide- and thromboxane-like activity as measured by the ability to produce contraction of vascular smooth muscle and aggregation of platelets under the same conditions described by others (5,6). The formation of platelet aggregating factors followed the time course previously reported (5), was blocked by indomethacin and was also inhibited by CO and metyrapone. This was demonstrated with two different series of

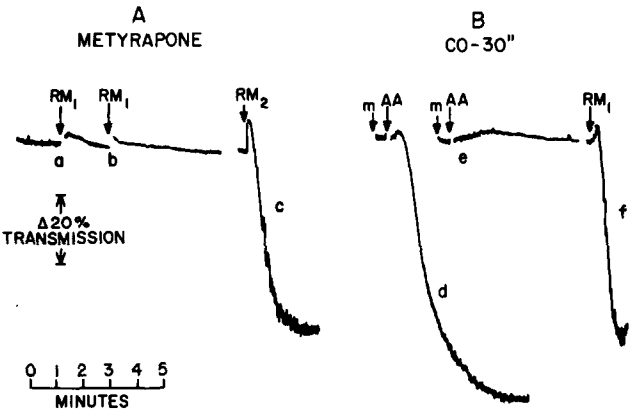

Figure 4. Effects of CO and metyrapone on generation of platelet aggregating factors from arachidonic acid by platelet microsomes. A: (a) (b) - 10μl arachidonic acid added to 25μl platelet microsomes (8mg/ml) containing 2.5mM metyrapone. After 10" at 37°C 0.5ml platelet suspension was added to the reaction mixture (RM_1), (c) RM_2-normal platelet microsomes (- metyrapone) incubated 10 sec. with arachidonic acid followed by addition of platelet suspension containing 0.6mM metyrapone. B: (d) 250μg microsomal protein (m) added to 0.5ml platelet suspension at 37°C. Arachidonic acid (AA) added to give concentration of 3μM, (e) same as (d), platelets treated with CO for 30 sec., (f) RM_1 (as above) added to CO-treated platelet suspension.

experiments. In one type of experiment platelet microsomes were incubated with arachidonic acid at 37°C with constant stirring. After 10 sec. platelet suspension (at 37°C) was added, and the light transmission recorded continuously. Under these conditions strong aggregation resulted. Platelet aggregation was blocked by pre-treatment of the microsomes with metyrapone (2.5mM) (fig. 4a,b). Increasing the arachidonate concentration in the incubation mixture 5-10 fold overcame the metyrapone inhibition. The concentration of metyrapone attained in the final platelet suspension (0.6mM) had little or no effect on the aggregation produced by adding preformed platelet aggregating factors produced from arachidonate by normal microsomes (fig. 4c).

In the second type of experiment microsomes were added to stirred platelet suspensions at 37°C in the absence of indomethacin. Subsequent

addition of 3μM arachidonic acid produced platelet aggregation (fig. 4d). However, in the absence of microsomes arachidonic acid did not induce aggregation below a concentration of 35μM. Pre-treatment of the microsomes with 15μM indomethacin also completely prevented platelet aggregation which was therefore clearly due to the generation of the platelet aggregating factors by the action of the exogenous microsomal enzyme activity on the low concentrations of added arachidonic acid. CO-treatment of platelet suspensions for 30 sec. prior to addition of microsomes strongly inhibited aggregation under these conditions (fig. 5e). However, when microsomes and arachidonic acid were preincubated together, apart from the platelets, and then added to CO-treated platelet suspensions aggregation was induced (fig. 5f). Although not shown in the figure, identical results were obtained when metyrapone replaced CO. In other words metyrapone and CO did not block the effect of directly added, preformed, platelet aggregating factors, but rather their formation from arachidonic acid, by platelet microsomes, was apparently inhibited.

DISCUSSION

These results conclusively demonstrate, for the first time, the presence of the hemoproteins, cytochromes P-450 and b_5 and their respective reductases in the platelet microsomal fraction. Two inhibitors (CO and metyrapone) of cytochrome P-450 were found to specifically block platelet aggregation induced by arachidonic acid. The effect of arachidonic acid is due to its biotransformation to endoperoxides and thromboxane A_2 by enzymes located in the microsomal fraction of platelets. The spectral evidence that platelet microsomal P-450 reacts with CO and metyrapone strongly suggests that these compounds could affect platelet aggregation by blocking P-450 catalyzed reactions. Inhibition of mitochondrial respiration in whole platelets by CO is unlikely to be the mechanism of action since, (1) we observed that rotenone was not an effective inhibitor

of arachidonate-induced platelet aggregation; and (2) inhibitors of mitochondrial electron transport have little effect on the metabolic pool of ATP, or the platelet release reaction, if glucose is present in the medium and glycolysis is unimpaired (20,21). Furthermore, CO and metyrapone cannot be acting as general metabolic poisons, or depressants of cellular activity, because maximal platelet aggregation could be elicited in their presence by A23187, ADP and sufficient concentrations of collagen. Indeed the platelet aggregating factors generated from arachidonic acid by microsomes in vitro were also able to cause aggregation of metyrapone and CO-treated platelets. Further work is in progress to determine what role, if any, cytochrome P-450 and cytochrome b_5 may play in platelet arachidonate metabolism.

Acknowledgments: This work was supported by NIH grants AM16678 and CA15897 (DLC) and HL18397 (MBF).

References

1. Hamberg, M. and Samuelsson, B. (1974) Proc. Nat. Acad. Sci. USA 71, 3400-3404.
2. Ho, P.P.K., Walters, C. P. and Hermann, R. G. (1976) Biochem. Biophys. Res. Comm. 69, 218-224.
3. Gerrard, J. M., White, J. G., Rao, G. H. R. and Townsend, D. (1976) Amer. J. Path. 83, 283-298.
4. Miyamoto, T., Ogino, N., Yamamoto, S. and Hayaishi, O. (1976) J. Biol. Chem. 251, 2629-2636.
5. Needleman, P., Moncada, S., Bunting, S., Vane, J. R., Hamberg, M. and Samuelsson, B. (1976) Nature 261, 558-560.
6. Needleman, P., Minkes, M. and Raz, A. (1976) Science 193, 163-165.
7. O'Brien, P. J. and Rahimtula, A. (1976) Biochem. Biophys. Res. Comm. 70, 832-838.
8. Hrycay, E. G. and O'Brien, P. J. (1971) Arch. Biochem. Biophys. 147, 14-27.
9. Hrycay, E. G. and O'Brien, P. J. (1972) Arch. Biochem. Biophys. 153, 480-494.
10. Kato, R., Iwasaki, K., Shiraga, T. and Noguchi, H. (1976) Biochem. Biophys. Res. Comm. 70, 681-687.
11. Feinstein, M. B. and Fraser, C. (1975) J. Gen. Physiol. 66, 561-581.

12. Omura, T. and Sato, R. (1964) J. Biol. Chem. 239, 2370-2378.
13. Phillips, A. H. and Langdon, R. G. (1962) J. Biol. Chem. 237, 2652-2659.
14. Strittmatter, P. (1967) Methods in Enzymol. 10, 561-565.
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
16. Miller, J. L. (1974) Ph.D. Thesis, University of Connecticut.
17. Orrenius, S., Ellin, A., Jakobsson, S., Thor, H., Cinti, D., Schenkman, J. and Estabrook, R. (1973) Drug Metab. and Dispos. 1, 350-357.
18. Leibman, K. C. (1969) Mol. Pharmacol. 5, 1-10.
19. Netter, K. J., Kahl, G. F. and Magnussen, M. P. (1969) Naunyn-Schmied. Arch. Pharmak. 265, 205-215.
20. Holmsen, H. and Day, H.J. (1971) Ser. Haemat. 4, 28-58.
21. Mills, D.C.B. (1975) in Biochemistry and Pharmacology of Platelets (Ciba Symposium 35) pp. 153-167.